Malaysian Applied Biology (2024) 53(3): 125-136
https://doi.org/10.55230/mabjournal.v53i3.2949

Research

Cytotoxic Effect of Bajong LN Rice Methanol Extract on Human Squamous Cell Carcinoma, ORL-48

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ABSTRACT

Oral squamous cell carcinoma (OSCC), a major oral cancer, significantly challenges treatments and impacts patient quality of life; current therapies often cause severe side effects, highlighting the urgent need for gentler alternatives. Rice stands as one of the primary cereal grains providing the daily caloric intake for more than half of the global population. Extensive research has demonstrated the significant health benefits derived from rice, attributed to its abundance of bioactive compounds. This study endeavours to explore the potential cytotoxic effects of Bajong LN rice, a pigmented purple rice indigenous to Sarawak, on human squamous cell carcinoma, ORL-48 cells. Cells were cultured in complete DMEM/F-12 media and incubated under standard culture conditions. Upon reaching 80% confluency, the cells were treated to varied concentrations (ranging from 0 µg/ml to 2000 µg/ml) of Bajong LN rice methanol extract (BLN-ME) and cisplatin. Subsequently, the cells were incubated for 48 and 72 hours, and their cytotoxicity was assessed using the MTS assay. Results demonstrated that cisplatin inhibited ORL-48 cells with an IC_{50} of 7.483 µg/ml and 3.877 µg/ml; and an IC_{80} of 40.649 µg/ml; and 17.543 µg/ml for 48 and 72 hours, respectively. Correspondingly, BLN-ME exhibited a notable cytotoxic effect against ORL-48 cells at 48- and 72-hour intervals, with an IC_{50} of 354.4 µg/ml and 342.0 µg/ml; and an IC_{80} of 450.3 µg/ml and 423.63 µg/ml, respectively. The cytotoxic activity of BLN-ME against ORL-48 cells was observed in both a time and dose-dependent manner. Morphological analysis and the Trypan blue exclusion assay corroborated the MTS assay's findings. Our preliminary findings provide the first scientific evidence of the cytotoxic effect of BLN-ME specifically against human squamous cell carcinoma, ORL-48 cells. This study suggests the potential of BLN-ME as a promising anti-cancer agent, presenting opportunities for further investigation into its underlying cytotoxic mechanisms.

Key words: Bajong LN rice, cisplatin, cytotoxicity, MTS assay, ORL-48 cells

Article History
Accepted: 3 May 2024
First version online: 30 September 2024

Cite This Article:
Ahmad, V.N., Mohamed, N.A.H., Amom, Z., Heah, K.G., Mohd Zain, N. & Amin, I.M. 2024. Cytotoxic effect of Bajong LN rice methanol extract on human squamous cell carcinoma, ORL-48. Malaysian Applied Biology, 53(3): 125-136. https://doi.org/10.55230/mabjournal.v53i3.2949

INTRODUCTION

Oral squamous cell carcinoma (OSCC), the most prevalent oral cancer form, poses significant treatment challenges. The intricate anatomy of the oral cavity and the aggressive progression of OSCC significantly impair patient quality of life, necessitating the urgent development of less invasive and more tolerable therapeutic approaches. Current treatment modalities, including surgery, radiation, and chemotherapy, often result in severe side effects and the risk of disfigurement, emphasizing the need for alternative strategies (González-Ruiz *et al*., 2023; Imbesi *et al*., 2023).

In this landscape, the exploration of plant crude extracts and their compounds in the quest for cancer therapy is garnering increasing interest, highlighting the potential medicinal benefits of these natural resources. One of the fascinating aspects concerning plants lies in the components derived from our daily dietary sources, such as rice (*Oryza sativa* L.). As a staple food for a significant portion of the global population, rice is not only a nutritional powerhouse but also a source of bioactive compounds with potential health benefits (Ren *et al*., 2023).

Bajong LN rice cultivar 'Sarawak', a unique variety from Sarawak, Malaysia, stands out due to its high anthocyanin content, attributed to its naturally deep purple color (Wen, 2015). Anthocyanins from plants have anticancer properties that include inducing apoptosis, inhibiting cancer cell proliferation, and preventing angiogenesis, underscoring their value in fighting cancer (Hui *et al*., 2010; Nascimento *et al*., 2022). Moreover, Bajong LN rice is enriched with vitamin E isomers, including α-tocopherol, β-tocopherol, and α-tocotrienol (Shammugasamy *et al*., 2015). Several studies have reported that vitamin E isomers are known for their antioxidative properties and their effectiveness in suppressing cancer cell proliferation and invasion, presenting another layer of potential anticancer activity (Dolfi *et al*., 2013; Yang *et al*, 2020). These components position Bajong LN rice as a viable candidate for exploring natural anticancer agents, due to its unique blend of bioactive compounds which may significantly against OSCC.

Furthermore, the notion that the Bajong LN rice cultivar 'Sarawak', Malaysia affects cancer cell proliferation is based on several previous studies involving other pigmented rice cultivars, which also indicate their cytotoxic and apoptotic effects on the various cancer cells. Hui *et al*. (2010) revealed significant cytotoxic and apoptotic effects of black rice cultivar 'Chinese' extract on various types of breast cancer cells. Another investigation focusing on the Payao purple rice cultivar 'Thailand' extract demonstrated cytotoxic activities on human hepatocellular carcinoma cancer cells (HepG2) (Banjerdpongchai *et al*., 2013). Rukmana *et al*. (2016) study, reported that extracts from three black rice bran variants cultivar 'Indonesia', exhibit cytotoxic effects on human Burkitt lymphoma (Raji) and HepG2 cancer cells. Further to this, Avanthi and Vigasini (2021) documented that the extract of defatted rice bran cultivar 'India' inhibits the viability of breast (MCF-7) and lung (A549) cancer cell lines. Hartati's (2022) study confirmed that the extract of the black rice cultivar 'Indonesia' significantly inhibits human breast cancer cells (T47D), with molecular evidence showing the extract induces apoptosis and cell cycle arrest. Because pigmented rice cultivars were found to affect the various cancer cell viability and proliferation, we hypothesized that Bajong LN rice may have a cytotoxic effect on OSCC.

Therefore, in this study, the cytotoxic effects of Bajong LN rice methanol extract (BLN-ME) on the ORL-48, OSCC cells were investigated by assessing cell viability and proliferation via the MTS assay. The cytotoxicity results were further validated with Trypan blue exclusion assay by using the obtained IC_{50} and IC_{80}, as well as observed the morphology of the cells by using an inverted light microscope. This preliminary investigation seeks to add to the understanding of BLN-ME's potential as a therapeutic agent against OSCC.

MATERIALS AND METHODS

Preparation of cell line

OSCC cell line, ORL-48, utilized in the study, was acquired through the collaborative efforts of Assoc Prof Dr Khor Goot Heah with Cancer Research Malaysia. ORL-48 cells were derived from a female patient diagnosed with a gum tumor. The ORL-48 cells exhibit characteristics typical of OSCC, including appearing as adherent with polygonal shape, epithelial morphology the ability to form tight, and cohesive monolayers upon growth. These cells were cultured in complete Dulbecco's modified Eagle medium/Nutrient F-12 (DMEM/F-12) media (Gibco, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, USA) and 1% antibiotics comprising 10,000 IU penicillin and 10 mg/mL streptomycin (10,000 U/mL) (Gibco, Gaithersburg, MD, USA). The cells were cultured as a monolayer and permitted to achieve approximately 80% confluence in a humidified incubator at 37°C with 5% CO_2 (Esco, Singapore), utilizing T25 and T75 flasks (Thermo Fisher Scientific Nunc, USA). The cells within passages 20-25 were carefully chosen and used to maintain their original phenotypic characteristics and growth vitality. This ensures the reliability of experimental outcomes by avoiding phenotypic drift or genetic instability that could occur in cells beyond this passage range.

Collection of Bajong LN rice

The Sarawak local rice sample used in this study was the whole grains of Bajong LN rice, purchased from Kenyalang Antares Enterprise and Sarawak Specials (Sarawak, Malaysia). They are sourced from local rice plantation sites located in Lubok Nibong within the Betong Division of Sarawak. The rice sample was immediately processed upon receipt to preserve its bioactive compounds, ensuring its freshness and integrity for the study. The rice sample was washed with autoclaved distilled water to eliminate any impurities or dirt. Subsequently, it was dried under sterile conditions in a food dryer (Morphy Richards, UK) at 40°C and then finely ground into a powder form. This finely ground powder was stored and shielded from light at 4°C until its use.

Preparation of Bajong LN rice methanol extract

The extraction procedure was carried out at the Faculty of Dentistry, UiTM Campus Sungai Buloh. The finely ground dried rice powder underwent maceration using methanol as the solvent (Sigma-Aldrich, USA). The powder was allowed to soak in the methanol solvent at room temperature for 72 hr, with a ratio of dried powder to methanol solvent set at 1:10 (10 g of rice powder: 100 mL methanol). This soaking process was repeated three times to ensure the maximum yield of methanol-soluble compounds from the rice. Subsequently, the mixture was filtered using Whatman filter paper No. 1 (Cytiva, USA), and the filtrate was subjected to drying procedures using a rotary evaporator (Buchi-R210, Switzerland) then followed by freeze-dried (Labconco, USA) to obtain dry extract for a period ranging from 72 to 96 hr. The final yield of methanol extract obtained was 3.75% [(weight of dried extract/weight of dried powder) x 100]. Finally, the extract was stored in a freezer at -20°C until utilized for subsequent experiments.

Preparation of test compounds

Bajong LN rice methanol extract (BLN-ME) and cisplatin (Sigma-Aldrich, USA) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) at a concentration of 50 mg/mL as the primary stock solution. The final working solutions of BLN-ME and cisplatin were diluted with culture media to ensure that the final concentration of DMSO in the cell culture was <0.1%. This DMSO percentage was deemed non-toxic to the cells. Cisplatin served as the positive control. Serial dilutions of BLN-ME and cisplatin were prepared accordingly.

Assessment of cytotoxicity via MTS assay

The effect of BLN-ME on ORL-48 cells was determined using an MTS assay kit (Abcam, UK). The kit is a colorimetric sensitive quantification of viable cells in proliferation and cytotoxicity assay. The method is based on the reduction of the MTS tetrazolium compound by viable cells to generate a colored formazan product that is soluble in cell culture media. This conversion is carried out by NAD(P)H-dependent dehydrogenase enzymes in metabolically viable cells. The formazan dye produced by viable cells can be quantified by measuring the absorbance at OD = 490 nm, which is proportional to the number of viable cells. To normalize the values, the background absorbance was first subtracted from all experimental readings. The background absorbance was measured from wells containing only the culture media and MTS reagent, without any cells. This procedure was implemented to ensure that the absorbance values reflected cellular metabolic activity accurately.

In the experimental design, ORL-48 cells were precisely seeded at a density of 1×10^4 cells per well in 96-well microplates (Thermo Fisher Scientific Nunc, USA), with a uniform volume of 100 µL of complete culture media per well. This meticulous approach guaranteed the formation of a consistent monolayer across the surface of each well, a crucial factor for uniform cell distribution and the establishment of optimal growth conditions. Following seeding, the cells were incubated in a humidified incubator with 5% CO_2 at 37°C for 24 hr to facilitate and confirm cell adhesion.

After 24 hr, the culture media was removed and the cells were treated with 100 µL of fresh culture media which contained different final concentrations of BLN-ME (ranging from 0-2000 µg/mL. Several controls were incorporated to ensure the reliability of the data: a negative control (untreated group), involving wells with cells but without the BLN-ME, was used to measure baseline cellular activity. A positive control, with wells containing cells treated with cisplatin (ranging from 0-2000 µg/mL), a cytotoxic agent widely used to treat OSCC, was established to validate the assay's sensitivity. A blank control, consisting of wells filled only with the culture media and MTS reagent, was utilized for background subtraction. In addition, a series of standards with known concentrations of the MTS tetrazolium compound were utilized to generate a calibration curve (data not shown), thus ensuring the assay's linearity and quantitative accuracy. The cells were incubated for both 48 hr and 72 hr respectively in a humidified incubator at 37°C with 5% CO_2. Multiple incubation times (48 & 72 hr) were employed to capture the time-dependent effects of BLN-ME on ORL-48 cells, facilitating the observation of both immediate and delayed cytotoxic responses. This approach is essential for comprehensively evaluating the anticancer activity of the extract. After the respective treatment periods, 10 µL of MTS reagent was added into each well and incubated again for an additional 3 hr in a humidified incubator at 37°C with 5% CO_2. The microplate was shaken for 1 min to homogenize its contents. The absorbance was measured at a wavelength of 490 nm and with a reference wavelength of 620 nm using a colorimetric microplate reader (Tecan, Infinite® 200 Pro). The viable cells were calculated using the following formula:

Equation 1:

$$\text{Percentage of viability (\%)} = \frac{\text{(Mean absorbance of sample - Mean absorbance of blank)}}{\text{(Mean absorbance of control - Mean absorbance of blank)}} \times 100\%$$

From the calculation generated by using the formula, the respective IC_{50} and IC_{80} values, which indicate the concentration of BLN-ME or cisplatin necessary to inhibit 50% and 80% of cell proliferation, were ascertained through the construction of dose-response curves. The percentage of cell viability was plotted against the logarithm of BLN-ME or cisplatin concentrations, and nonlinear regression analysis (specifically sigmoidal dose-response curve fitting) was conducted using GraphPad Prism version 9.0 software. The software automatically calculated the IC_{50} and IC_{80} based on the curve that was fitted. The respective IC_{50} and IC_{80} concentrations determined in this assay were used in the subsequent experiments. The respective IC_{50} and IC_{80} concentrations determined in this assay were used in the subsequent experiments.

Morphological observation

The morphological examination of ORL-48 cells subjected to treatment with BLN-ME and cisplatin was conducted simultaneously with the Trypan blue exclusion assay. Following the incubation periods, cells were washed with 1X phosphate-buffered saline (PBS) solution (Gibco, Gaithersburg, MD, USA) and then observed under an inverted light microscope at 100x magnification (Nikon Eclipse Ts2), and images were captured using a Nikon camera. Observations focused on general indicators of cell health and viability, such as changes in cell density, shape, and adherence properties. This approach allowed for a preliminary evaluation of cytotoxic effects based on visible alterations compared to untreated control cells. The subsequent step of the Trypan blue exclusion assay was then initiated by the protocol.

Trypan blue exclusion assay

Trypan Blue exclusion (TBE) assay was employed to quantitatively assess cell viability following BLN-ME treatment. This assay distinguishes between viable and non-viable cells based on membrane integrity: viable cells exclude the dye and remain unstained, while non-viable cells take up the dye and appear blue. The proportion of unstained (viable) cells to the total number of cells was calculated, providing an estimate of BLN-ME's cytotoxicity.

In the experimental design, the ORL-48 cells were seeded at 4×10^5 cells per well in 6-well plates (Thermo Fisher Scientific Nunc, USA), with a uniform volume of 2 mL of complete culture media. Following seeding, the cells were incubated in a humidified incubator with 5% CO2 at 37°C for 24 hr to facilitate and confirm cell adhesion. After 24 hr, the cultured media was removed and the cells were treated with 2 mL of fresh complete culture media which contained respective IC_{50} and IC_{80} concentrations of BLN-ME. Both IC_{50} and IC_{80} concentrations were obtained from the MTS assay. To ensure data reliability, several controls were implemented: the negative control, an untreated group receiving only complete culture media without BLN-ME, was established to define baseline cellular activity and morphology. Additionally, a positive control involved wells with cells treated with cisplatin at its IC_{50} concentration, confirming the assay's sensitivity. The cells were incubated for 72 hr respectively in a humidified incubator at 37°C with 5% CO_2.

Following morphological observation, the cells were washed with 1X PBS solution. The solution was removed and subjected to trypsinization. After the addition of 2 mL fresh media, the suspension was centrifuged at 1500 rpm for 5 min, and the supernatant was aspirated. The cells pellet was resuspended in 1 mL of fresh culture media, and subsequently, 10 µL of this suspension was mixed with 10 µL of trypan blue dye solution (Sigma-Aldrich, USA). Cell counting was performed using the Countess 3 automated cell counter (Thermo Fisher Scientific, USA) and the results were recorded.

Statistical analysis

All data outcomes from the experiments were reported as mean ± standard deviation (SD) using GraphPad Prism version 9.0 software (GraphPad Software, USA). Each experiment was performed in triplicate and repeated three times. IC_{50} and IC_{80} were derived from sigmoidal dose-response curves, with software automatically calculating these values from logarithmic concentration plots. R squared value reflects the proportion of variance in the dependent variable explained by the independent variable(s), indicating model fit quality. R squared values approaching 1 suggest a high explanatory power; values above 0.7 are considered strong; 0.5 to 0.7 moderate; and below 0.5 indicative of a weak correlation. The differences between groups were evaluated using one-way ANOVA tests, followed by Dunnet's post-hoc test. Statistical significance was defined at $*p<0.05$; $**p<0.01$.

RESULTS AND DISCUSSION

Cytotoxicity effect of BLN-ME against ORL-48 cells

The ability of cytotoxic agents to eliminate cancer cells plays a crucial role in cancer therapy. To the best of our knowledge, this study is the first to investigate the cytotoxic impact of Bajong LN rice on

OSCC, particularly targeting ORL-48 cells. As detailed in Table 1, BLN-ME demonstrated significant antiproliferative effects on ORL-48 cells, although with higher IC_{50} and IC_{80} values compared to cisplatin, indicating its potential as an alternative therapeutic agent.

Table 1. IC_{50} and IC_{80} concentrations for BLN-ME and cisplatin at 48 and 72 hr of incubation time

Treatment:	IC_{50} (µg/mL)		IC_{80} (µg/mL)	
	48 hr	72 hr	48 hr	72 hr
BLN-ME	354.40 ± 0.04	342 ± 0.05	450.3 ± 0.02	423.63 ± 0.04
Cisplatin	7.48 ± 0.03	3.88 ± 0.07	40.65 ± 0.09	17.54 ± 0.04

The cytotoxic activity of BLN-ME against ORL-48 cells was evidenced by the observed reduction in cell viability and proliferation at both 48 hr (as shown in Figure 1) and 72 hr (illustrated in Figure 2) post-incubation. In Figure 1, there is a discernible decrease in cell viability correlating with escalating doses of BLN-ME over a 48-hr incubation period (p<0.05). The baseline cell viability for ORL-48 cells, as represented by the untreated group, was recorded at 100%. At a concentration of 354.4 ± 0.04 µg/mL, BLN-ME achieved an IC_{50} value, significantly diminishing cell viability compared to the control (p<0.05). Additionally, as the dosage of BLN-ME was progressively elevated, a corresponding decline in cell viability ensued, with the 450.3 ± 0.02 µg/mL concentration of BLN-ME inducing the IC_{80} value in this experiment (p<0.05).

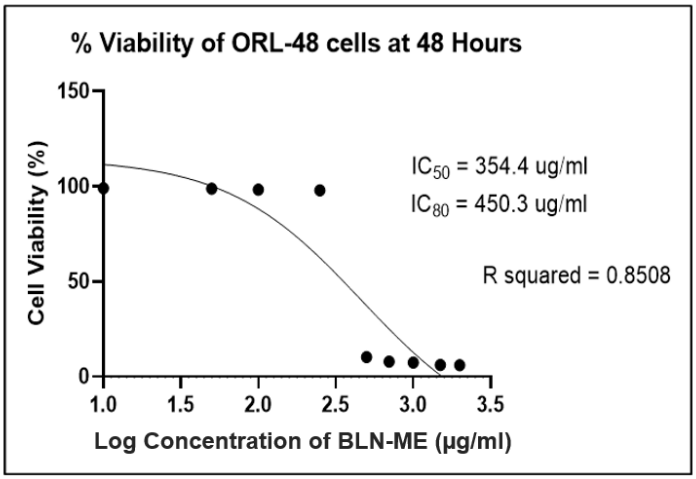

Fig. 1. Graph for percentage viability of ORL-48 cells treated with BLN-ME at 48 hr.

Figure 2 demonstrates the efficacy of BLN-ME over a 72-hr treatment period. An inverse relationship is observed between the percentage of cell viability and increasing doses of BLN-ME. As the dosage of the treatment escalates, there is a corresponding decrease in cell viability compared to the control (p<0.05). There is a discernible variation in the IC_{50} values over the incubation periods of 48 and 72 hr. Specifically, the IC_{50} value at 72 hr is 342 ± 0.05 µg/mL, representing a marginal decrease of 1.04-fold from the value recorded at 48 hr (354.4 ± 0.04 µg/mL). This underscores the influence of incubation time on the effectiveness of the treatment dosage. Similarly, the IC_{80} (423.63 ± 0.04 µg/mL) observed over the 72 hr treatment period also differs from the IC_{80} (450.3 ± 0.02 µg/mL) for the 48 hr duration. This variation further highlights the impact of extended incubation time on the potency of the treatment as well as indicating that a lower concentration over 72 hr is more effective than a higher concentration over 48 hr.

The observed variations in the IC_{50} and IC_{80} values for BLN-ME over different incubation periods, specifically for 48 hr and 72 hr, can be influenced by several factors. One such factor that has been discussed in previous studies, is the temporal dependency of cellular response (Fallahi *et al*., 2013; Webb *et al*., 2015). It is acknowledged that the reactivity of cells to cytotoxic agents varies over time. In this study, we speculated that the prolonged exposure of ORL-48 cells to the bioactive constituents in the BLN-ME for 72 hr may enhance their interaction, thereby exerting a more noticeable effect at a slightly reduced concentration.

Apart from Bajong LN rice, numerous cancer-related studies have demonstrated various rice varieties from different countries exhibit cytotoxic and apoptotic effects on human cancer cell lines. Hartati's (2022) study revealed that the aqueous extract of the black rice cultivar 'Indonesia' significantly inhibits human breast cancer cells (T47D), cervical cancer cells (HeLa) and sarcoma cells (U2OS)

with molecular evidence showing the extract induces apoptosis and cell cycle arrest on the cancer cells. Rukmana *et al*. (2016) conducted a preliminary study, demonstrating that ethanolic extracts from three black rice bran variants, including the 'Indonesia' cultivar, and one white rice bran variant exhibit cytotoxic effects on human Burkitt lymphoma (Raji) and HepG2 cancer cell lines. Banjerdpongchai *et al*. (2013) discovered that HepG2 cells show significant cytotoxicity when treated with the methanolic extract of the 'Thailand' purple rice cultivar. This study also confirmed that the rice extract induces apoptosis through the mitochondrial pathway. Hui *et al*. (2010), demonstrated the effectiveness of an anthocyanin-rich extract from the 'Chinese' black rice cultivar against various breast cancer cell lines, (MCF-7, MDA-MB-231, and MDA-MB-453), which exhibited the highest sensitivity, with an IC_{50} value obtained within 24 hr in an MTT assay. Furthermore, research by Uttama *et al*. (2010) investigated the chloroform extract from the white rice bran 'Hommali 105' 'Indonesia 'cultivar', which significantly inhibited prostate cancer cells (PC3), followed by HeLa and MCF-7 cells. Notably, their extract did not display cytotoxic activities against either lung cancer cells (CORL23) or normal cells (MRC5). Our study aligns with those findings by demonstrating the cytotoxicity effect of the rice. Nonetheless, it is crucial to recognize that the IC_{50} values exhibit variability across different studies. Such discrepancies are reflective of the unique geographical origins, soil physiochemical properties, and cultivation practices associated with each rice variety, which in turn influence their phytochemical compositions and resultant biological activities (Zhou *et al*., 2014; Kumarathilaka *et al*., 2018).

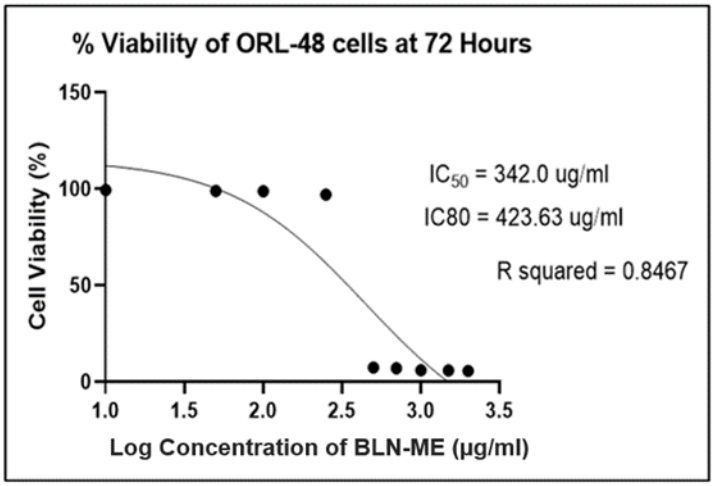

Fig. 2. Graph for percentage viability of ORL-48 cells treated with BLN-ME at 72 hr.

Additionally, the discrepancy in IC_{50} values across different cancer cell lines stands as a significant factor (Weinstein & Lorenzi, 2013; Brown *et al*., 2022). Furthermore, it is important to note that the efficacy of a crude extract and its active compound in eliciting a cytotoxic effect can differ significantly. This discrepancy is primarily attributed to the concentration and purity of active compounds. Crude extracts typically comprise a mixture of various substances (Perrut & Perrut, 2018), including the active ingredients responsible for the cytotoxicity, as well as other compounds that may be inactive or less potent.

In Figure 3, the finding revealed that cisplatin significantly exhibited the inhibitory effects on ORL-48 cells over a 48-hr incubation period. At a concentration of 7.48 ± 0.03 µg/mL, cisplatin achieved an IC_{50} value, significantly diminishing cell viability compared to the control (p<0.05). Additionally, as the dosage of cisplatin was progressively elevated, a corresponding decline in cell viability ensued, with the 40.65 ± 0.09 µg/mL concentration of cisplatin inducing the IC_{80} value (p<0.05).

The study utilizes cisplatin as the positive control, acknowledged for its efficacy in treating head and neck squamous cell carcinoma. Its administration, however, is linked to several chronic, dose-dependent side effects (Falco *et al*., 2021). It has been established that lower concentrations of cisplatin induce apoptosis, whereas higher concentrations result in necrosis (Sancho-Martinez *et al*., 2011). Current research efforts are directed towards augmenting the efficacy of cisplatin through combination chemotherapy. This involves exploring its synergistic potential with other agents to enhance patient safety and mitigate adverse effects (Kasiram *et al*., 2021). Cisplatin, in line with its known chemotherapeutic attributes, displayed significantly greater antiproliferative effectiveness compared to BLN-ME. However, accumulating evidence from various studies underscores the cytotoxic impact of cisplatin on normal

human cells, thereby raising substantial concerns about its use as an anticancer agent.

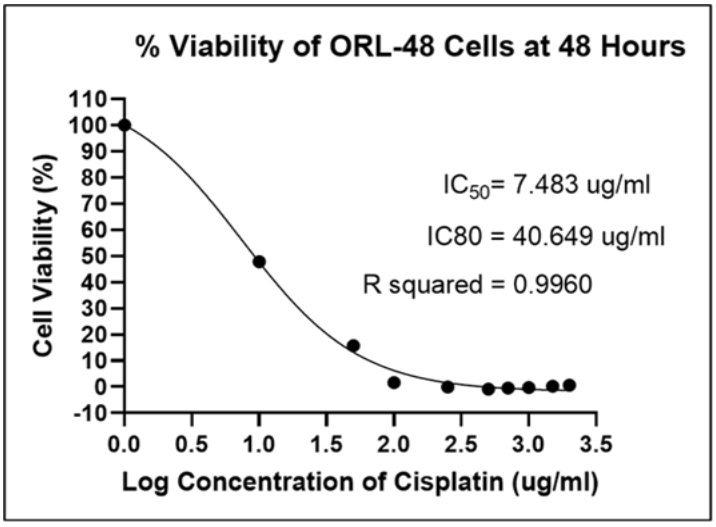

Fig. 3. Graph for percentage viability of ORL-48 cells treated with cisplatin at 48 hr.

In Figure 4, the enhanced efficacy of cisplatin in eradicating ORL-48 cells with an extended incubation period of 72 hr is demonstrated (p<0.05). It is observed that the IC_{50} value, recorded at 3.88 ± 0.07 µg/mL for the 72-hr duration, is 1.93 times lower than that for 48 hr (p<0.05). Additionally, the IC_{80} value at 72 hr, being 17.54 ± 0.04 µg/mL, markedly differs from the 48-hr value, with the former being 2.32 times lower than the latter (p<0.05).

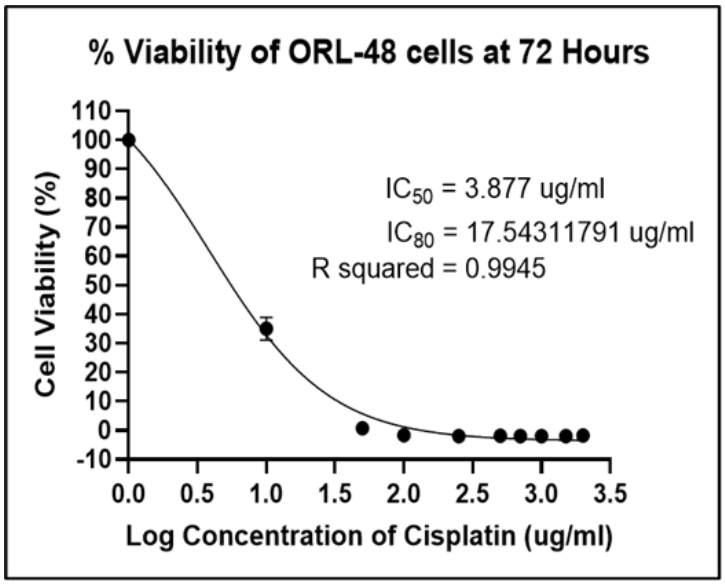

Fig. 4. Graph for percentage viability of ORL-48 cells treated with cisplatin at 72 hr.

Morphological analysis on the viability of ORL-48 cells using an inverted light microscope

Morphological analysis provides qualitative data that complements the quantitative insights derived from the MTS assay. It allows for the evaluation of cellular morphology and can provide information on cellular responses and differentiation processes (Molinari *et al*., 2003). The MTT or MTS assay, on the other hand, is a widely used method for assessing cellular metabolic activity and toxicity (Ghasemi *et al*., 2021). By combining the two approaches, researchers can gain a more comprehensive understanding of cellular behavior and function. For example, the MTT or MTS assay can provide quantitative data on cell viability and metabolic activity, while morphological analysis can provide visual evidence of cellular changes and differentiation (Ishikawa *et al*., 2019). This combination of qualitative and quantitative data can enhance the interpretation of experimental results and provide a more complete picture of cellular

responses to various treatments or conditions. In addition, through microscopic examination of changes in cell structure and integrity, researchers are also able to corroborate the results of the MTT or MTS assay.

For this study, the IC_{50} of cisplatin and the IC_{50} as well as IC_{80} of BLN-ME were selected, respectively, from the MTS assay results obtained over a 72-hr incubation period. An inverted light microscope was utilized to identify key differences in cell appearance.

In Figure 5, the microscopic images depict the morphological changes in cell growth, shape, and pattern density across each treated group. Following a 72 hr incubation period, the treated cells displayed a significant reduction in confluence, transitioning from the untreated ORL-48 cells' typical polygonal morphology (as shown in Figure 5a) to rounded shape and detached forms (as shown in Figure 5b & 5c). This transformation indicates a marked decrease in cell-cell adhesion, with cells in treated groups appearing almost singularly, commonly seen in cells undergoing cytotoxic stress and hindering cell proliferation. Conversely, cells in the untreated group (Figure 5a) maintained their regular, adherent morphology, layering upon each other, a condition conducive to healthy cell growth or proliferation. The untreated group (Figure 5a) proliferated significantly, nearly reaching maximum confluency. Furthermore, cells treated group (as shown in Figure 5d) displayed a significant reduction in confluence and showed an increase in apoptotic-like features, such as cell shrinkage and membrane blebbing, even in the absence of Scanning Electron Microscopy (SEM) or Transmission Electron Microscopy (TEM) for resolution enhancement. These observations suggest the cytotoxic effects of BLN-ME and cisplatin on ORL-48 cells, indicating their role in apoptosis induction and reduced cell viability.

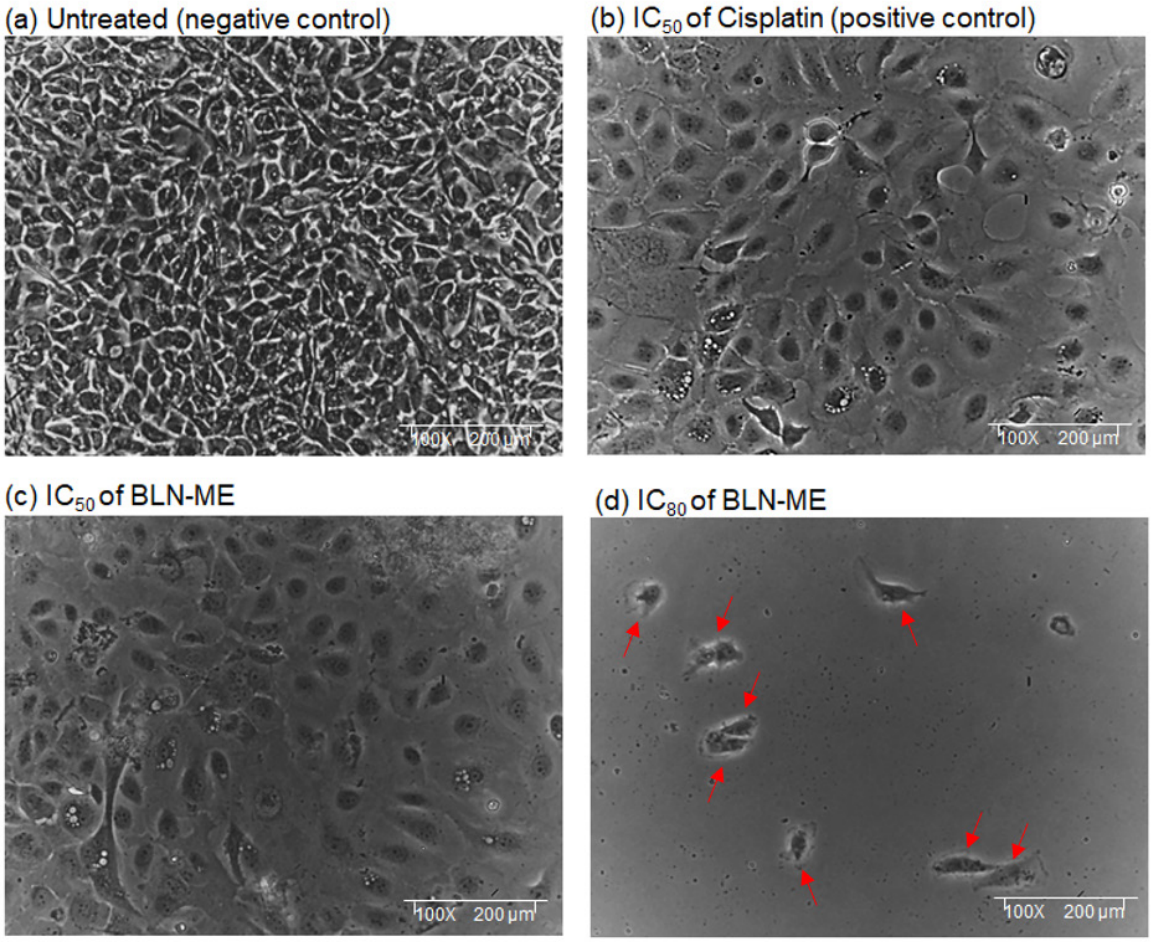

Fig. 5. A representative set of microscopic images of ORL-48 cells at 100X magnification with (a) untreated cells as a negative control; (b) IC_{50} of cisplatin; (c) IC_{50} of BLN-ME; and (d) IC_{80} of BLN-ME. The apoptotic-like features are highlighted by the red arrows in the images.

Trypan blue exclusion (TBE) assay analysis

TBE assay is based on the principle that dead cells absorb trypan blue dye into their cytoplasm, while live cells remain unstained. It is one of the earliest methods for detecting the cytotoxicity of a treatment and continues to be a relevant assay for confirming the results of the MTT or MTS assay (Pei

et al., 2020). Fundamentally, the TBE assay relies on the integrity of the cellular membrane (Piccinini *et al*., 2017; Ude *et al*., 2022), in contrast, the MTT or MTS assay is colorimetric assay which is based on the conversion of MTT or MTS reagent into purple formazan crystals by living cells, which is determined by the mitochondrial activity of viable cells (Bahuguna *et al*., 2017). In this study, the TBE assay was specifically utilized to validate the cytotoxic outcomes derived from the MTS assay. Upon conducting morphological analysis, the influence of both BLN-ME and cisplatin on the viability of ORL-48 cells was promptly verified and quantified via the TBE assay.

In Figure 6, the viability of ORL-48 cells treated with the IC_{50} of cisplatin was reduced to 53.00 ± 2.65%, compared to the untreated group. For the IC_{50} of BLN-ME, cell viability decreased to 48.30 ± 2.04%. The IC_{80} of BLN-ME markedly demonstrated a substantially lower viability percentage, at 18.67 ± 3.51%. The consistent outcomes were observed in cells treated with BLN-ME at its IC_{50} and IC_{80}, as well as cisplatin at its IC_{50}, mirroring the results obtained from the MTS assay. These findings strongly affirm that BLN-ME significantly impedes the viability and proliferation of ORL-48 cells. Notably, the treatment with BLN-ME at the IC_{80} concentration resulted in a substantial reduction in cell count. The coherence of these findings with the MTS assay highlights the detrimental effect of BLN-ME on ORL-48 cells, reinforcing its potential as an inhibitor of cell proliferation. This preliminary observation lays crucial groundwork for more exhaustive future studies into the cytotoxic mechanisms affecting ORL-48 cells and for uncovering the mechanisms underlying the observed cytotoxicity.

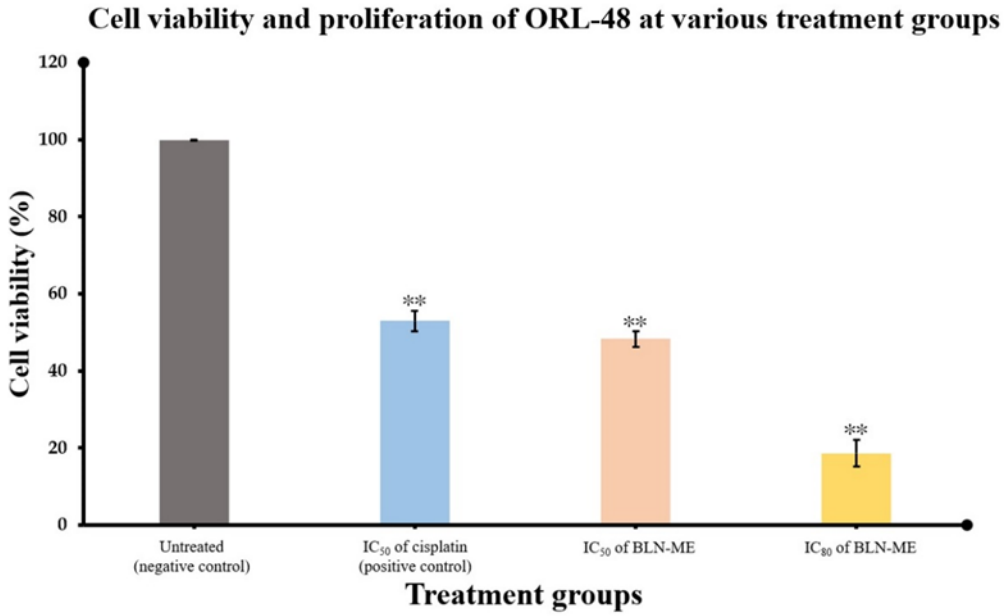

Fig. 6. The effect of BLN-ME and cisplatin on the viability and proliferation of ORL-48 cells. Data represent the mean ± SD; ** P<0.01 compared with the untreated group (negative control).

There are noteworthy aspects to discuss and propose about the study of rice crude extract and its IC_{50} value. This topic merits attention due to its significant implications in the field of phytochemical research, particularly in the context of rice-based studies. This primary study's exploration into the cytotoxic effects of BLN-ME on ORL-48 cells has culminated in an IC_{50} value of 342 ± 0.05 µg/mL. This finding, while higher than the frequently cited benchmark of less than 20 µg/mL for crude extracts (Geran *et al*., 1972 ; Chen *et al*., 1988), resonates with the evolving trends in phytochemical research, particularly those centered around rice-based studies. Notably, the IC_{50} value we report meticulously corroborated through the MTS assay, microscopic analysis, and TBE assay, underscores a distinct cytotoxic potency inherent to the purple rice extract. In the broader context, our findings are consistent with parallel studies from regions like Indonesia (Uttama *et al*., 2010; Rukmana *et al*., 2016; Hartati, 2022), China (Hui *et al*., 2010), Thailand (Banjerdpongchai *et al*., 2013) and India (Avanthi & Vigasini, 2021). These studies have reported IC_{50} values well above the 300 µg/mL mark, with some extending up to 2000 µg/mL across diverse cancer cell lines. Such findings are indicative of a broader, emerging pattern where rice extracts, despite their higher IC_{50} values, demonstrate a significant capability to induce apoptosis and cell cycle arrest (Hui *et al*., 2010; Banjerdpongchai *et al*., 2013; Hartati, 2022). This observation is crucial in reevaluating the therapeutic potential of these extracts beyond the conventional cytotoxicity benchmarks. The prevalent adherence to a less than 20 µg/mL threshold for cytotoxicity in cancer cell lines seems to be a legacy of historical criteria, likely stemming from guidelines established

by the National Cancer Institute (NCI) in the 1970s and 1980s, as referenced by Chen *et al*. (1988); and Geran *et al*. (1972) then cited by many studies that exploring the cytotoxic effects of crude extracts from medicinal plants up to the present day (Zulkapli & Abdul Razak, 2019; Canga *et al*., 2022; Asenye *et al*., 2023). Efforts were undertaken to obtain the latest and specific NCI guidelines related to the IC_{50} dosages for plant extracts, as referenced in the cited literature; however, there was no available information regarding these detailed guidelines. As a result, they remained unattainable. Thus, this long-standing benchmark, while formative, may not comprehensively capture the complexities and nuances of current phytochemical research, especially when considering extracts derived from daily dietary staples like rice. The chemical and biological distinction of rice extracts, when compared to traditional medicinal plant parts such as leaves, roots, stems, or bark, is striking. Rice, as a staple food consumed daily, presents a unique interaction with biological systems, which could justify the higher IC_{50} values observed in studies like ours.

The methodological rigor of our study, evident in the application of various analytical assays, bolsters the credibility of our findings. These methods collectively affirm the cytotoxic effect of the rice extracts, emphasizing the necessity for context-specific evaluation criteria in phytochemical research. The disparity in cytotoxicity values between rice extracts and those derived from more conventional medicinal plants highlights the need for a nuanced approach to evaluating the anticancer potential of different phytochemical sources. Our study not only contributes to the expanding body of research on the anticancer properties of rice extracts but also prompts a reconsideration of the existing cytotoxicity evaluation framework.

In reflecting on the limitations of our research, we acknowledge its focus was solely on the ORL-48 cells, without comparison to normal cell lines, thus limiting insights into the selective cytotoxicity of BLN-ME. The study's exclusive use of an inverted light microscope, while effective for initial observations, limits our capacity to discern more detailed apoptotic features that SEM or TEM analyses could reveal. Additionally, relying solely on methanol maceration for extraction might not capture the full spectrum of bioactive components in Bajong LN rice, hinting at the need for a broader range of extraction methods to uncover its full anticancer potential.

For future studies, it is essential to include normal human gingival fibroblasts and gingival keratinocytes to ascertain BLN-ME's specificity and safety. Investigating the molecular pathways involved in apoptosis induction specifically through both the intrinsic and extrinsic routes of the apoptosis pathway, as well as examining cell cycle arrest and other cell death mechanisms such as autophagy or necroptosis, will provide a more comprehensive understanding of BLN-ME's anticancer mechanisms. Expanding the OSCC cell line spectrum and employing diverse extraction techniques are crucial steps forward in fully assessing BLN-ME's therapeutic potential.

CONCLUSION

Overall, our findings provide initial scientific evidence that BLN-ME inhibits the growth and proliferation of ORL-48 cells in a dose-dependent manner. This study pioneers the examination of Bajong LN rice from Sarawak, Malaysia, as a novel anticancer resource. Highlighting the untapped therapeutic potential of regional rice varieties, our research emphasizes the critical role of biodiversity agricultural product s in identifying innovative health solutions. It is important to acknowledge that our study was limited to a single cancer cell line and utilized only one extraction method, which may restrict the generalizability of our results. Further research is necessary to investigate the effects of BLN-ME across a broader spectrum of oral cancer cell lines and to employ various extraction methods to ascertain the full scope of its anticancer potential. Such studies will help elucidate the pathway involved in apoptosis and other cell death mechanisms such as autophagy or necroptosis that underpin the effects of BLN-ME on ORL-48 cells. A thorough investigation into the active constituents of BLN-ME is also crucial, as the specific compounds mediating these effects are yet to be identified.

ACKNOWLEDGEMENTS

We would like to extend our sincere appreciation to Cancer Research Malaysia for generously providing ORL-48 cells. We would also like to thank Kenyalang Antares Enterprise and Sarawak Specials (Sarawak, Malaysia) for continuously managing and selling samples of Bajong LN rice. Additionally, we are grateful to the Faculty of Dentistry and the Faculty of Health Sciences at Universiti Teknologi MARA (UiTM) for their invaluable support and the provision of necessary facilities that were crucial for the successful conduct of this study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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